activation of the voltage sensors in the fourth transmembrane segment (S4) is transduced into pore opening via coupling of the S4-S5 linker to the C-terminal S6 segment. In TRPA1, the gain-of-function mutation N855S located in the S4-S5 region has been associated with familial episodic pain syndrome. In an attempt to elucidate the role of the S4-S5 linker and its putative interaction(s) with S6 or the first C-terminal helix in the voltage-dependent gating of TRPA1, we used site-directed mutagenesis, whole-cell electrophysiology, single-channel recording, and molecular dynamics simulations. The charge-reversal mutations K868E and K969E resulted in a decrease in the rectification index compared to wild-type TRPA1 channels, and a virtually voltage-independent conductance-voltage (G-V) relationship. This effect was also observed in the adjacent charge-neutralizing mutant H970A, but was less pronounced in charge- reversal H970D. These results indicate that positively charged residues in the S4-S5 linker and the helix adjacent to the C-terminal S6 segment play a vital role in the voltage-dependent gating of TRPA1.

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Ultraviolet Light Phototransduction Activates TRPA1 to Mediate Melanin Synthesis in Human Melanocytes

Nicholas W. Bellono, Laura G. Kammel, Anita L. Zimmerman, Elena Oancea.
Brown University, Providence, RI, USA.

Human skin is constantly exposed to solar ultraviolet radiation (UVR), the most prevalent environmental carcinogen. Humans have the unique ability among mammals to respond to UVR by increasing their skin pigmentation, a protective process driven by melanin synthesis in epidermal melanocytes. However, the mechanism used by melanocytes to detect and respond to UVR is not well understood. Here we report that transient receptor potential A1 (TRPA1) ion channels expressed in human melanocytes are activated by UVR and mediate early melanin synthesis. We show that in human epidermal melanocytes (HEMs) physiological doses of UVR activate a retinal-dependent current mediated by TRPA1. The UVR photocurrent density was reduced by TRPA1 antagonists and abolished in HEMs expressing TRPA1-targeted miRNA. The TRPA1 photocurrent is UVA specific and requires G protein signaling, providing the first evidence for TRPA1 function in mammalian phototransduction. In HEMs, TRPA1 activation contributes to UVR-induced calcium responses to mediate downstream cellular effects. Remarkably, the UVR-induced and retinal-dependent early increase in cellular melanin content was significantly reduced in HEMs treated with TRPA1 antagonists and abolished in HEMs expressing TRPA1-targeted miRNA, suggesting that TRPA1 is required for early melanin synthesis. Our results show that TRPA1 is essential for a novel extra-ocular phototransduction pathway in human melanocytes that is activated by physiological doses of UVR and results in early melanin synthesis.

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Characterization of Small Molecule TRPC3 and TRPC6 agonist and Antagonists

Xiaoping Xu[1], Irina Lozinskaya[1], Melissa Costell[1], Zuojun Lin[1], Jennifer A. Ball[2], Roberta Bernard[1], David J. Behm[1], Joseph P. Marino[1], Christine G. Schnackenberg[1].
[1]GlaxoSmithKline, King of Prussia, PA, USA, [2]Bristol University, Bristol, United Kingdom.

Canonical transient receptor potential channels (TRPC3/6) are mechano-sensitive, receptor- and store-operated channels that mediate Ca^{2+}/Na^{+} influx into cells. TRPC3/6 are implicated in the regulation of vascular tone, cell growth, proliferation and pathological hypertrophy. Potent and selective small molecule TRPC3/6 agonist and antagonist tools had been lacking to study the functions of TRPC3/6. We report here potent, small molecule agonist (GSK1702934A) and antagonists (GSK417651A and GSK2293017A) of TRPC3/6. Whole-cell patch-clamp experiments demonstrated that GSK1702934A activated TRPC3 and TRPC6 current in HEK293 cells transduced with recombinant human TRPC3 or TRPC6 with an EC_{50} of ~ 0.08 μM and 0.44 μM, respectively. GSK417651A inhibited both TRPC3 and TRPC6 current with IC_{50}s of ~ 0.04 μM. A more potent TRPC3/6 antagonist GSK2293017A exhibited an IC_{50} of ~ 0.01 μM. GSK417651A was likely acting on TRPC3/6 from the extracellular side, because dialyzing the cells with 10 μM GSK417651A in the pipette solution did not prevent the activation of TRPC6. In the rat cardiomyocyte like cell line H9C2, GSK1702934A (1 μM) activated TRPC6-like current which was completely blocked by GSK417651A (1 μM). TRPC3/6 activator GSK1702934A (1 μM) transiently increased the perfusion pressure of isolated rat heart retrogradely perfused via aortic cannulation. This effect was completely eliminated by pretreatment with the TRPC3/6 blocker GSK2293017A (1 μM). IV bolus of GSK1702934A (0.3 ~ 3 mg/kg) transiently increased blood pressure by 15 ~ 35 mmHg in conscious Sprague Dawley rats. This blood pressure effect was dose-dependently inhibited by pretreatment of GSK2293017A (2.5 ~ 250 μg/kg/min infusion for 30 min) with complete inhibition occurring at a free plasma concentration of 0.81 μM. Therefore, these small molecule agonist and antagonists could be useful tools to help understand the characteristics and functions of TRPC3/6 in tissues and animals.

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PIP_2 Dynamics underlying Muscarinic or Vasopressin Receptor-Activated TRPC3 C6 And C7 Currents

Kyohei Itsuki[1,2], Yuko Imai[1], Yasushi Okamura[3], Ryuji Inoue[2], Masayuki X. Mori[2].
[1]Kyushu University, Faculty of Dental Sciences, Fukuoka, Japan, [2]Fukuoka University, Dept. of Physiology, Fukuoka, Japan, [3]Osaka University, School of Medicine, Dept. of Physiology, Osaka, Japan.

Subfamily of human expressed TRPC channels (TRPC3/6/7) are activated by 'diacylglycerol' (DAG), a phospholipase C (PLC) hydrolyzed product of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] (Hofmann *et al*., 1999, Nature). In contrary, we have recently reported that the depletion of $PI(4,5)P_2$ by-itself act as a potent negative regulator to all these channels even in the presence of DAG (Imai *et al*., 2012, J. of Physiol.). Stimulation upon the vasoconstrictile receptors coupled with PLC theoretically causes inseparable bimodal effect to these TRPC channels, i.e. activation and inhibition by DAG production and coincident $PI(4,5)P_2$ reduction or depletion which is corresponding to the strength of PLC activities. Here, to elucidate such self-limiting regulatory function coupled to $PI(4,5)P_2$-DAG signal, we simultaneously measure TRPC6/7 currents in the whole-cell configuration and $PI(4,5)P_2$ dynamics by FRET using $PI(4,5)P_2$ binding PH-domain sensor proteins in the various strength of muscarinic- or vasopressin-receptor stimulation. Our simultaneous detection approach reveals good kinetics correlation between TRPC activation/fast-inactivation and $PI(4,5)P_2$ depletion. Furthermore, a simple self-limiting regulation model wherein experimentally determined $PI(4,5)P_2$ binding constants incorporated aware an emergence of fast recovery of $PI(4,5)P_2$ to produce slow inactivation (plateau phase) of TRPC currents. We find that such model implicated $PI(4,5)P_2$ enhancement can be reproduced after the fast $PI(4,5)P_2$ depletion under the robust agonist stimulation by the local FRET measurement near the channels. Hence, these data indicates that self-limiting regulation coupled to $PI(4,5)P_2$-DAG signal is the pivotal mechanism to understand the receptor-PLC mediated TRPC3/6/7 channels activity.

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RASD1 Independently Activates TRPC4 through Gαi of GPCR

Jinhong Wie, Insuk So.
School of medicine Seoul National University, Seoul, Korea, Republic of.

Canonical transient receptor potential (TRPC) channels have six transmembrane (6-TM) domains and are Ca^{2+}-permeable and non-selective cation channels. It is generally speculated that TRPC channels are activated by stimulation of Gq-PLC-coupled receptors and oxidation. Activator of G-protein signaling1 (AGS1 or RASD1), the ras-related protein, interacts with Gi/Go and activates heterotrimeric G-protein signaling systems independent of G-protein-coupled receptor (GPCR). It is previously reported that AGS1 is related to GIRK channel and Ca^{2+} channel. However it is unknown whether AGS1 is associated with TRPC channels. We assumed that AGS1 might regulate TRPC4 channel, since AGS1 interacts with Gi/Go and TRPC4 is activated by Gi/o subunits. Here, we measured whole cell current of TRPC4/5 after the co-expression of TRPC4 or TRPC5 with constitutively active form of small GTPases in HEK293 cells. AGS1 (CA) mutant (Q to L) activated TRPC4 (38.8 ± 7.2 pA/pF) without GTPγS and independently of GPCR. Pertussis toxin (PTX), $G\alpha_i$ specific inhibitor, blocked RASD1-activated TRPC4 current (3.4 ± 1.6 pA/pF). When co-expressed with dominant negative $G\alpha_i$ protein subtype, TRPC4 activation by RASD1 was completely inhibited. With previous report that TRPC4 are activated primarily by selective Gαi subunits rather than $G\alpha_q$, these results suggest that AGS1 activates TRPC4 channel through modulating Gαi subunits and AGS1 is a new activator for TRPC4 channel.

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An Essential Role of $PI(4,5)P_2$ for Maintaining the Activity of the Transient Receptor Potential Canonical (TRPC)4 Channel TRPC4β

Hana Kim, Insuk So.
Seoul National University College of Medicine, seoul, Korea, Republic of.

The Transient Receptor Potential Canonical 4 (TRPC4) channel is a Ca^{2+}-permeable, non-selective cation channel in mammalian cells and mediates

a number of cellular functions. Many studies show that TRPC channels are activated by stimulation of $G\alpha_q$-PLC-coupled receptors. However, our previous study showed that the TRPC4 current was inhibited by a constitutively active form of $G\alpha_q(G\alpha_q^{Q209L})$. It may have caused a shortage of phosphoinositide phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) because $G\alpha_q^{Q209L}$ would have persistently activated PLCβ. Therefore, we used an inducible system to regulate $PI(4,5)P_2$ specifically and acutely. The TRPC4β current was reduced by inducible $G\alpha_q^{Q209L}$ but not by the mutants whose binding ability to PLCβ is impaired. If the aforementioned phenomenon resulted from desensitization of TRPC4 by protein kinase C (PKC), the current of TRPC4β (T877A) mutant which is not phosphorylated by PKC would not be inhibited by co-expression of a $G\alpha_q^{Q209L}$. However, we detected the inhibitory action of $G\alpha_q^{Q209L}$. Depletion of $PI(4,5)P_2$ using the inositol polyphosphate 5-phosphatase (Inp54p) inducible system led to an irreversible inhibition of TRPC4 currents after application of rapamycin to HEK293 cells co-expressing TRPC4 with Inp54p. On the other hand, phosphatidylinositol 4-phosphate 5-kinase (PIP5K) inducible system did not activate the initial gating of TRPC4β. Even in the case of $G\alpha_{i2}$-activated TRPC4β currents, the acute depletion of $PI(4,5)P_2$ led to reduced TRPC4β currents. Therefore, we suggested that $PI(4,5)P_2$ is not the activator for TRPC4 activation but it is still necessary for regulating TRPC4 activation. Especially, TRPC4 desensitization might be a result of hydrolysis of $PI(4,5)P_2$ since TRPC4 desensitization through muscarinic receptor 3 which activates $G\alpha_q$-PLC pathway disappeared by adding $PI(4,5)P_2$ and nonhydrolysis $PI(4,5)P_2$. These findings indicate an essential role of $PI(4,5)P_2$ for maintaining the activity of TRPC4β.

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Stretch-Induced Activation of NFAT Signaling in Hl-1 Cardiomyocytes

Shaymaa A. Bahnassy[1], Michael Poteser[2], Hannes Schleifer[2], Christoph Romanin[3], Klaus Groschner[2].
[1]Institute of Pharmaceutical Sciences - Pharmacology and Toxicology, University of Graz, Graz, Austria, [2]Institute of Biophysics, Medical University of Graz, Graz, Austria, [3]Institute of Biophysics, University of Linz, Graz, Austria.

Mechanical forces are efficiently converted into cellular signals to initiate structural and functional remodeling of cardiac myocytes. Molecular mechanisms underlying cardiac mechanotransduction are still barely understood. Nuclear Factor of Activated T-cells (NFAT) is an important calcium-responsive transcription factor and a key player in cardiac remodeling processes. In this study, we investigated the involvement of Ca^{2+} signaling molecules as a link between mechanical stretch and NFAT translocation in HL-1 atrial myocytes. We specifically focused on the potential role of receptor-operated and/or store-operated Ca^{2+} permeable channels of the TRPCs (classical transient receptor potential) and the Orai family. Populations of HL-1 cells overexpressing GFP-NFAT were exposed to a single-stretch by 20% for 20 minutes on commercially available silicon membranes and NFAT localization was then analyzed by fluorescence microscopy. Results revealed that stretched HL-1 cells significantly increased NFAT nuclear translocation when compared to unstretched cells. Similar NFAT activation was obtained when HL-1 cells were stimulated by either endothelin-1 (100 nM) or thapsigargin (1μM) in the absence of a stretch stimulus. These results demonstrate that similar levels of NFAT activation are induced in HL-1 cells by either mechanical stress, cell activation via Gq-coupled receptors or depletion of internal Ca^{2+} stores. HL-1 cells were found to express several TRPC isoforms that might contribute to mechanotransduction. Our data suggest a linkage between mechanical stretch and cardiac remodeling involving Ca^{2+} entry and NFAT activation.

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2328-Pos Board B347

Investigation of the ADP-Ribose-Dependent Gating Mechanism of the TRPM2 Channel

Balázs Tóth, László Csanády.
Semmelweis University, Budapest, Hungary.

TRPM2 is a non-selective Ca^{2+} permeable cation channel, expressed abundantly in brain, immune cells, and pancreatic β-cells. Under physiological conditions it might play a role in glucose-induced insulin secretion, and in the immune response. Besides, it enhances the sensitivity of cells toward oxidative stress induced damage. Thus, under pathological conditions, TRPM2 is responsible for neuronal cell death during ischemia/reperfusion in the brain.

The TRPM2 channel is co-activated by intracellular Ca^{2+} and ADP-ribose (ADPR). ADPR binds to the C-terminal NUDT9-H domain, while the Ca^{2+} binding sites have not yet been identified. Intriguingly, when expressed in isolation, the NUDT9-H domain functions as an active ADPR hydrolase which breaks ADPR into AMP and ribose-5-phosphate. In a previous study we showed that Ca^{2+} dependent gating is well described by the Monod-Wyman-Changeux model. However, little is known about the mechanism by which binding, and possibly hydrolysis, of ADPR is coupled to channel gating. Steady-state single-channel kinetic analysis could be very useful for addressing such details. However, wild-type TRPM2 channels are not amenable to such studies, because they inactivate irreversibly shortly after patch excision, precluding collection of sufficient numbers of gating events. Recently, we have identified that inactivation is caused by a conformational change of the selectivity filter, and can be completely prevented by a triple mutation in this region. This pore mutant TRPM2 channel (T5L), which mimics the filter sequence of TRPM5, retains unabated maximal activity for over 1 hour. Although the mutations result in altered permeation properties, ADPR- and Ca^{2+}-dependent gating remains intact.

Presently, we are investigating the ADPR-dependent gating mechanism of this mutant channel by steady-state single-channel kinetic analysis. Measurements are underway.

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TRPM2 Channels Protect Hearts from Ischemia-Reperfusion Injury

Joseph Y. Cheung[1], JuFang Wang[1], Erhe Gao[1], Jianliang Song[1], Xue-Qian Zhang[1], Madesh Muniswamy[1], Karthik Mallilankaraman[1], Shu-jen Chen[2], Iwona Hirschler-Laszkiewicz[2], Kathleen Conrad[2], Kerry Keefer[2], Arthur M. Feldman[1], Barbara A. Miller[2].
[1]Temple University School of Medicine, Philadelphia, PA, USA, [2]Pennsylvania State University College of Medicine, Hershey, PA, USA.

TRPM2 channels are expressed in the heart and vasculature. To elucidate the function of TRPM2, we generated a global TRPM2-KO mouse. To evaluate whether TRPM2 channels were functional in the heart, we activated TRPM2 channels with H_2O_2 and demonstrated that Ca^{2+} influx was dependent on extracellular Ca^{2+} and significantly higher in WT compared to TRPM2-KO myocytes. We then examined the effects of TRPM2 channels on cardiac function, both at rest and after ischemia-reperfusion (I/R) injury. At rest, there were no differences in LV mass, heart rate, fractional shortening (FS) and +dP/dt between WT and TRPM2-KO hearts. At 2-3 days post-I/R injury, despite similar areas-at-risk and infarct sizes, TRPM2-KO hearts had lower FS and +dP/dt when compared to WT hearts. Compared to WT I/R myocytes, expression of Na^+/Ca^{2+} exchanger (NCX1) and NCX1 current were increased, and action potential duration was prolonged in TRPM2-KO I/R myocytes. After 2 h of hypoxia followed by 30 min of reoxygenation to simulate I/R in vitro, levels of reactive oxygen species (ROS) were significantly higher in TRPM2-KO when compared to WT myocytes. Oxygen radical scavenging enzymes (SOD1 and SOD2) and their upstream regulators (FoxO1, FoxO3a and HIF-1α) were lower while NADPH oxidase (Nox2) was higher in TRPM2-KO I/R hearts. We conclude that TRPM2 channels protected hearts from I/R injury by enhancing expression of oxygen radical scavenging enzymes, thereby reducing oxidative stress associated with ischemia-reperfusion.

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Mechanism of TRPM7 Channel Inhibition by 2-Aminoethyl Diphenyl Borinate (2-APB)

Rikki Chokshi, **J. Ashot Kozak**.
Wright State University, Dayton, OH, USA.

2-APB is a widely used chemical compound in ion channel research. It inhibits numerous channels that include inositol 1,4,5-trisphosphate receptors, store-operated calcium channels, connexins and TRP family cation channels TRPC3, TRPC5, TRPC6, TRPM2, TRPM3 and TRPM7. Native and overexpressed TRPM7 channels are inhibited by 2-APB with IC50-s in the 70-170 μM range. A characteristic of TRPM7 channels is their inhibition by intracellular Mg^{2+} and acidic pH. using patch-clamp electrophysiology, we recorded native TRPM7 channel activity in Jurkat T lymphocytes and tested 2-APB at 10-300 μM for its ability to inhibit TRPM7 currents. When internal HEPES buffer concentration was low (1 mM), 100-300 μM 2-APB inhibited 60-70% of TRPM7 current with a slow time course. This inhibition was voltage-independent and reversible. Increasing the pH buffering capacity of internal solution to 140 mM HEPES abolished the inhibitory 2-APB effect. Simply making the internal recording solution alkaline, greatly diminished 2-APB inhibition. using single-cell pHi imaging with the fluorescent pH indicator BCECF, we found that at concentrations of 100 μM and higher, 2-APB potently acidifies the cytoplasm. A similar 2-APB-induced acidification was also observed in HEK293 cells, often used for TRPM7 channel overexpression. In contrast to 2-APB, its analog 2,2-diphenyltetrahydrofuran (DPTHF) did not produce cytosolic acidification, when applied at concentrations of 150-300 μM. We also found that Kv1.3 channels expressed in Jurkat T cells are modulated by 2-APB: the apparent voltage dependence of these